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Local inflammation as a possible mechanism of resistance to gastrointestinal nematodes in Angus heifers

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Abstract

Understanding mechanisms of resistance to gastrointestinal nematodes is important in developing effective and sustainable control programs. A resource population of Angus cattle consisting of approximately 600 animals with complete pedigree records has been developed. The majority of these animals were completely characterized for their resistance to natural challenge by gastrointestinal nematodes. As the first step towards understanding the molecular basis of disease resistance, we investigated expression profiles of 17 cytokines, cytokine receptors, and chemokines using real-time RT-PCR in animals demonstrating resistance or susceptibility to pasture challenge. The animals exposed to natural infection for approximately 6 months were treated to remove existing parasites and then experimentally challenged with both *Ostertagia ostertagi* and *Cooperia oncophora*. The mRNA expression profiles of these genes in abomasal and mesenteric lymph nodes (ALN, MLN), fundic and pyloric abomasa (FA, PA), and small intestine (SI) were compared between resistant and susceptible animals. Resistant heifers exhibited elevated expression of inflammatory cytokines such as TNFα, IL-1β, and MIP-1α in fundic and pyloric abomasa 7 days post infection. Expression levels of IL-10, polymeric immunoglobullin receptor gene (PIGR), and WSX-1 were also 2.7–19.9-folds higher in resistant than susceptible heifers in these tissues. No difference in expression of CXCL6, CXCL10, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-12 p40, IL-13, IL-15 and IL-18 was observed between the two groups. The expression of MIP-1α, IL-6, and IL-10 was also elevated in small intestines in resistant animals. In contrast, little difference in expression of these genes was detected between resistant and susceptible groups in the draining lymph nodes. These data indicate that resistant animals can better maintain inflammatory responses at the site of infection, suggesting a possible novel mechanism of resistance.
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Keywords: Bovine; Nematodes; Gene expression; Cytokines; Chemokines; Resistance

1. Introduction

Gastrointestinal nematodes pose a serious problem to the American cattle industry with an estimated annual loss greater than $2 billion because of decreased animal productivity and increased treatment costs. Current nematode control strategies involve periodic administration of anthelmintics. The treatment is expensive and labor intensive and reduces profitability. In addition, complete reliance on anthelmintics has resulted in several negative impacts. First and foremost is the appearance of parasites resistant to anthelmintics throughout the world (Prichard, 1994).

Alternative nematode control strategies using herd management and immunological tools such as vaccines or immunomodulators have been exploited. Herd management has proven to be effective but costly.

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Vaccines or immunomodulators have had limited success (Gasbarre and Canals, 1989). At the present time the value of this approach is limited by the ability of nematodes such as *Ostertagia* to evade host immune responses (Gasbarre, 1997). Another major problem with this approach is the need for the treatment to confer protection against all important parasite species in a given locality while at the same time falling within producer guidelines regarding cost and labor. Because of these limitations, utilization of host resistance and genetic diversity in the host genome as an alternative to anthelmintics seems very attractive (Sonstegard and Gasbarre, 2001).

Studies have shown that the IL-4/IL-13 pathway protects against intestinal nematodes in mice, *Nippostrongylus brasiliensis* and *Trichinella spiralis*, by activating STAT6 via IL-4 receptor α (IL-4Rα), which in turn enhances intestinal smooth muscle contractility and increases intestinal mucus secretion to expel the parasites (Finkelman et al., 2004). Different parasites elicit different mechanisms for protective immunity. For example, host protection against *N. brasiliensis* requires $CD4^+$ T cells, while protection against *T. spiralis* requires both $CD4^+$ T cells and mast cells (Ha et al., 1983). Mast cells require a Th-2 environment for differentiation and proliferation and IL-4 promotes these processes as well as migration. Mast cells express proteases such as tryptases and chymases, which may make local niches unhabitable to intestinal parasites and mice deficient in mMCP-1, a chymase, were unable to expel *T. spiralis* (Knight et al., 2000). Mast cells even directly affect the fecundity of *N. brasiliensis* (Newlands et al., 1995). Recently, it was discovered that an increase in epithelial cell turnover rate in the mouse intestine acts like an ‘‘epithelial escalator’’ to expel *T. spiralis* (Cliffe et al., 2005). This increased cell turnover is under immune control by IL-13 and the chemokine CXCL10. Evidence also suggests that helminths alter host mucosal and systemic immunity, inhibiting dysregulated inflammatory responses in animal models and they could be used as therapeutic agents to treat inflammatory bowel disease in humans (Weinstock et al., 2005). While IL-4 is directly involved in protective immunity in rodents, IL-4 may not be associated with the protective mechanism against *Ostertagia ostertagi* in cattle (Almeria et al., 1998). Unlike rodent models, expulsion as a major protective mechanism has not been documented in the cattle–*Ostertagia* system, and overall, little is known about the mechanisms of resistance in cattle. In this study, we attempt to dissect mechanisms that contribute to resistance and protective immunity that may impact the development of alternative control strategies.

2. Materials and methods

2.1. Animals, parasites, and statistic analyses

Ten Angus heifers used in this experiment were from a resource population of Angus cattle (Gasbarre et al., 2001). To date, this population has approximately 600 animals. Among these animals, 479 have been fully characterized for parasite resistance. The breeding program to generate this population involved selected matings of cattle selected for high or low fecal eggs counts (EPG). At weaning, calves were placed on pastures infected with the two common nematode parasites, *O. ostertagi* and *Cooperia oncophora*, as well as *Nematodirus helvetianus* and *Trichuris* sp. The calves were kept on pastures from April to October. The calves were monitored weekly for fecal EPG using the zinc sulfate flotation method and serum pepsinogen level (Gasbarre et al., 1996). Monthly weight and hip height measurements were also recorded throughout the experiment (before the challenge described below). Based on actual weekly EPG counts for all animals on the study (Fig. 1A) and sire expected progeny difference (EPD) values for EPG, a total of 10 heifers were chosen for this study. Four of the 10 heifers used in this study were classified as susceptible (with high EPG EPD) and the remaining 6 were resistant (with low EPG EPD).

At the end of the grazing season (October 25, 2005), all 10 heifers were treated with a combination of anthemintics (10 mg fenbendazole and 0.5 mg moxidectin per kg of body weight) to remove existing parasites from exposure to parasites on infected pastures. After resting for 30 days on concrete to preclude further parasite exposure, the heifers were orally infected with both *O. ostertagi* and *C. oncophora* L3 larvae (10^5 larvae each). The infective L3 larvae were obtained from cultures maintained at the USDA-ARS Beltsville facilities. The heifers were handled according to the protocol approved by The USDA-ARS Animal Care and Use Committee following Institutional Animal Care and Use Committees (IACUC) guidelines. The heifers were killed at either 3 or 7 day post infection (DPI). Tissues collected included the draining lymph nodes of both abomasum (ALN) and small intestine (MLN), full thickness folds from the fundic abomasa (FA) and full thickness samples (i.e. from mucosal to serosal surfaces from the pyloric abomasa (PA), and small intestine (SI). SI tissues were collected from the jejunum approximately 5 m from the pyloric sphincter. Each target area was sampled in three to five distinct areas. In the case of the SI samples, care was taken to not include areas containing Peyer’s

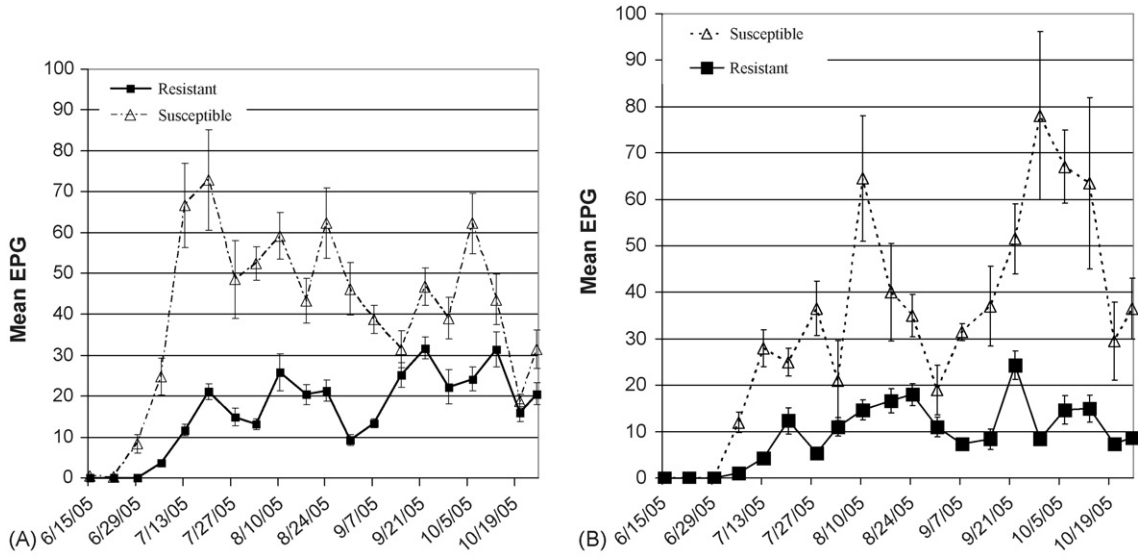

Fig. 1. (A) Mean fecal egg counts (EPG) of all resistant ($n = 22$) and susceptible ($n = 19$) Angus cattle on test. (B) Mean fecal egg counts (EPG) of resistant and susceptible Angus heifers ($n = 10$) selected for analysis in this study. Animals were generated from selective breeding based on EPG phenotypes, and, after weaning, were maintained on an infected pasture. Weekly EPG values were obtained using the zinc sulfate flotation method. Error Bars represent S.E.M.

Patches. Upon excision tissue samples were minced into 1–2 cm pieces and snap frozen in liquid nitrogen prior to storage at −80 C until total RNA was extracted.

Mean EPG, mean serum pepsinogen levels, total weight gains, and total hip gains were analyzed using Student's *t*-test with $P < 0.05$ being taken as significant. The data were not log transformed.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted using Trizol following the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Trace genomic DNA in the crude total RNA samples was removed by incubation with 4–6 units DNase I per 100 μg total RNA (Ambion, Austin, TX) at 37 °C for 30 min. Total RNA was further purified using an RNeasy Mini kit (Qiagen, Valenica, CA). The concentration of the total RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) and RNA integrity was verified using a Bioanalyzer 1000 (Agilent, Palo Alto, CA). The cDNA synthesis was performed with an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA).

2.3. Real-time RT-PCR

Table 1 lists the primers for 17 genes analyzed in this study. Real-time RT-PCR analysis was carried out with the iQ SYBR Green Supermix kit (Biorad) using 200 nM of each amplification primer and the first-strand cDNA (100 ng of the input total RNA equivalents) in a 25 μl reaction volume as described (Li et al., 2006). The amplification was carried out on an iCycler iQ™ Real-Time PCR Detection System (BioRad) with the following profile: 95 °C—60 s; 40 cycles of 94 °C—15 s, 60 °C—30 s, and 72 °C—30 s. The melting curve analysis was performed for each primer pair. The amplicons of selected genes were sequenced to verify their identities. Expression levels of ribosomal protein S29 (RPS29) was used as an endogenous control because the expression level of this gene remained constant across all experimental samples (mean cycle threshold ± S.D. = 19.91 ± 0.18). Relative gene expression data was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The fold change was calculated using the susceptible group as control.

3. Results

3.1. EPG and serum pepsinogen levels

Mean EPG values from the six resistant heifers were 9.6 while the four susceptible heifers had mean EPG values of 33.8 at the conclusion of the 6-month grazing period (Table 2). The difference is statistically significant ($P < 0.05$). Although there was an expected weekly fluctuation in EPG counts, in both the entire test population (Fig. 1A) and in the heifers used in this

Table 1
Primers used in the experiment

Accession	Forward primer	Reverse primer	Gene
AF348421	CCTCACCCACACCATCAG	GCGATCTCCCTTCTCCAG	TNFα
AF149249	GTGAGAGAGCTGCGTTGTG	TCCAGACAGACTTCCCTTCC	CXCL6
AY077840	AAGCCCACACTCCGTCTC	GGTCTCAAAATAGTCAGCTACG	MIPα
BC102879	AATGTCTACTCTCTCCTGTGAG	CATCATGTCCTGGAACACTTC	IL-18
NM174143	TGTCATCAACCGAGTCAAG	CCAGGGAACAGTCAAAGG	PIGR
NM173921	ACGCTGAACATCCTCACAAC	GCTCCTGTAGATACGCCTAAG	IL-4
NM173923	GTCTTCAAACGAGTGGGTAAAG	TGACCAGAGGAGGGAATGC	IL-6
NM173925	AAACACATTCCACACCTTTCC	AGCAGACCTCGTTTCCATTG	IL-8
NM174086	TAGGCAAGTCTATGGGATTTC	GCATTCATTACATCATCAAGTG	IFNγ
NM174088	CAAGCCTTGTCGGAAATG	AGATGTCAAACTCACTCATG	IL-10
NM174090	TCCATCCAGTGCTACTTGTG	ACTGCATCGCTGTTACTTTG	IL-15
NM174093	TCAGAATGGAAACCCTCTCTC	GCATGGATCAGACAACAGTG	IL-1β
NM180997	AAGTGAAGTCATTGCTGCTG	TGTCCATTGAATCCTTGATCTC	IL-2
XM592397	TTGGAAAGTTCTGTCAGTTGTC	TGTGGCAGGCTGAAGTTG	WSX-1
XM866821	TTCTCAGTCAATGTGCTAAGTC	AGCAGATTGTGTTATTGGTTTG	CXCL10
NM174089	ATGGTGTGGAGCCTCAAC	CTCGGACGTACTCACTGG	IL-13
U11815	ACCTCAGACCAGAGCAGTGA	CCCTCCTGACACTCCACTGT	IL-12 p40
BC102702	GGAGCCATCCGAGAAAATTCG	CAACTTAATGAAGCCGATGTCCTT	RPS29

study (Fig. 1B), resistant cattle demonstrated constantly lower EPG than those of susceptible animals. At the conclusion of the grazing period, resistant heifers had statistically significant higher serum pepsinogen levels than susceptible heifers ($P < 0.01$; Table 2).

3.2. Weight and hip height gains

Resistant heifers had higher weight and hip height gain over the experiment period (Table 2). However, the differences were not statistically significant.

3.3. Gene expression analyses

A total of 17 cytokines, cytokine receptors, and chemokines were analyzed for their gene expression using real-time RT-PCR (Table 1). All genes were detected in all of the tissues. The data were analyzed using four comparisons: (1) resistant versus susceptible at 3 DPI; (2) susceptible heifers at 7 DPI versus 3 DPI; (3) resistant heifers at 7 DPI versus 3 DPI; and (4) resistant versus susceptible at 7 DPI.

Table 2
A comparison between resistant and susceptible Angus heifers*

	Resistant	Susceptible
EPG (eggs per gram)	9.6 ± 0.9**	33.8 ± 10.5
Pepsinogen (mU)	1176.6 ± 104.3***	654.5 ± 69.4
Weight gain (lbs)	5.3 ± 10.5	−9.3 ± 5.0
Hip height gain (cM)	5.8 ± 1.7	4.8 ± 0.6

* Mean ± S.E.M.
** Significant at $P < 0.05$ based on unpaired Student's t-test.
*** Significant at $P < 0.01$ based on unpaired Student's t-test.

At 3 DPI, the L3 larvae have just completed the movement to the primary infection sites (abomasum and small intestine for *Ostertagia* and *Cooperia*, respectively). As expected, no genes were over-expressed in the resistant animals compared to the susceptible animals in these tissues. However, in the draining lymph nodes, IL-8 (in ALN) and IL-6 (in MLN) were up-regulated slightly (2.3- and 2.6-folds, respectively), in the resistant heifers at this time point. Similarly, in the susceptible groups, no genes were up-regulated at 7 DPI when compared to 3 DPI in FA, PA, and SI. The patterns of gene expression comparing resistant heifers at 7 DPI versus 3 DPI and resistant versus susceptible at 7 DPI were almost identical. The results of the comparison between resistant and susceptible heifers at 7 DPI are presented in detail. The results presented were based on fold changes using 2-fold as a cutoff.

Comparing resistant to susceptible animals at 7 DPI, no genes showed changes greater than 2-fold in both abomasal and mesenteric lymph nodes. In fundic abomasa, TNFα, MIP-1α, IL-1β, IL-10, polymeric immunoglobullin receptor gene (PIGR) (Fig. 3), and WSX-1 (Fig. 4) were all up-regulated greater than 2-fold in resistant animals. The gene expression patterns in pyloric abomasa were very similar to those in fundic abomasa: TNFα, MIP-1α, IL-1β, IL-10, PIGR, and WSX-1 were also up-regulated in resistant heifers at 7 DPI. In small intestine, MIP-1α, IL-6 and IL-10 were up-regulated greater than 2-fold in resistant heifers at 7

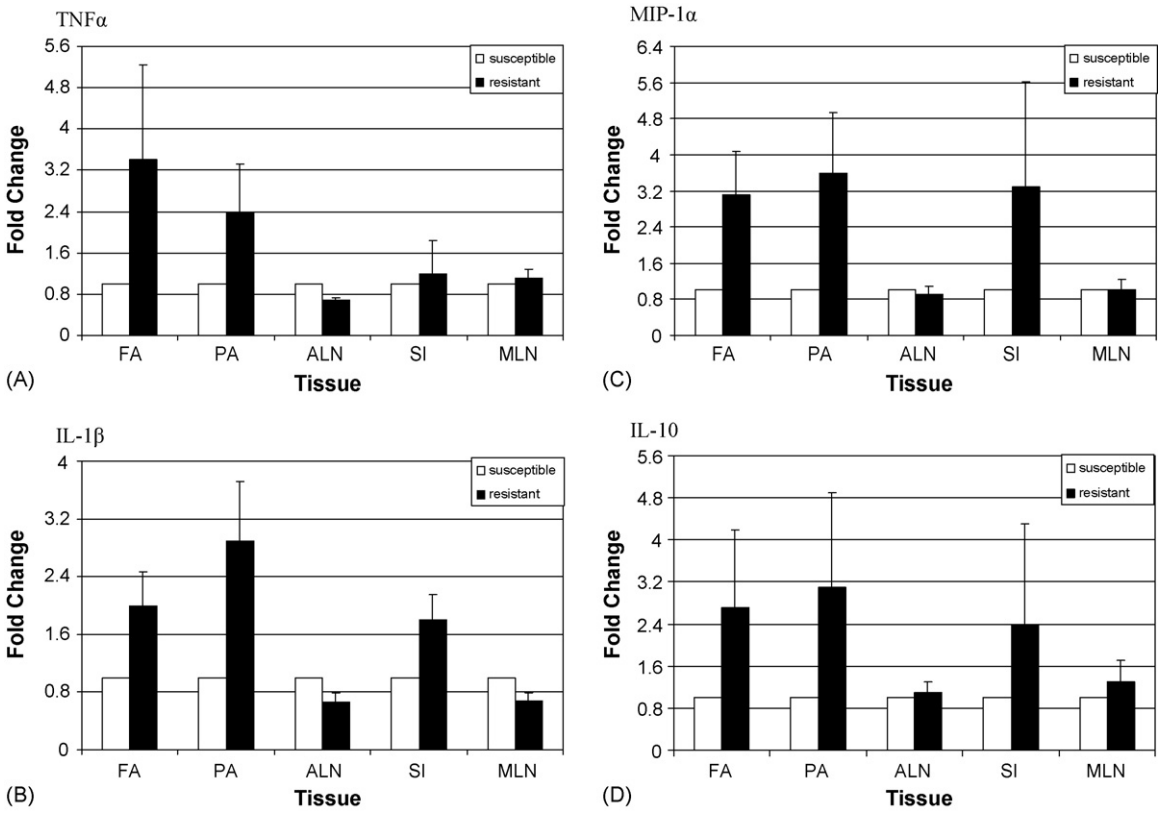

Fig. 2. The relative gene expression of inflammatory cytokines and chemokines at 7 DPI. Mean fold changes ($n = 3$) were presented with S.E.M. as error bars. The expression levels in the susceptible heifers were arbitrarily assigned as 1.0. (A) TNFα; (B) IL-1β; (C) MIP-1α; and (D) IL-10. ALN: abomasal lymph node; MLN: mesenteric lymph node; FA: fundic abomasum; PA: pyloric abomasum; and SI: small intestine.

DPI. All other genes did not show any differences between the two groups.

The expression levels of inflammatory cytokines and chemokines (TNFα, MIP-1α, IL-10, and IL-1β) across all five tissues were presented in Fig. 2. Although IL-6 plays a key role in inflammatory response, this cytokine was only up-regulated in the small intestine. Polymeric immunoglobullin receptor gene was over-expressed

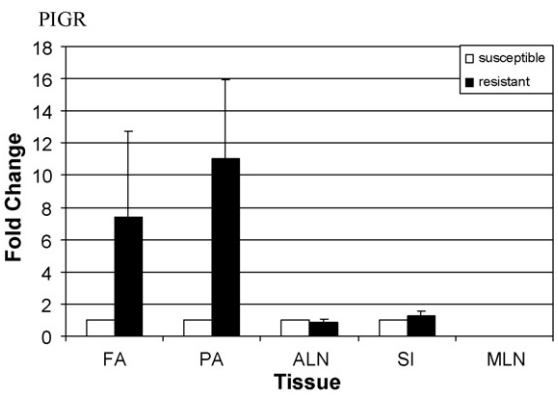

Fig. 3. The relative gene expression of PIGR at 7 DPI. Mean fold changes ($n = 3$) were presented with S.E.M. as error bars. The expression levels in the susceptible heifers were arbitrarily assigned as 1.0. ALN: abomasal lymph node; MLN: mesenteric lymph node; FA: fundic abomasum; PA: pyloric abomasum; and SI: small intestine.

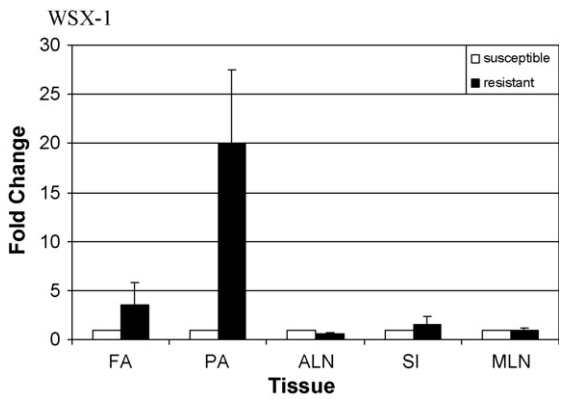

Fig. 4. The relative gene expression of WSX-1 at 7 DPI. Mean fold changes ($n = 3$) were presented with S.E.M. as error bars. The expression levels in the susceptible heifers were arbitrarily assigned as 1.0. ALN: abomasal lymph node; MLN: mesenteric lymph node; FA: fundic abomasum; PA: pyloric abomasum; and SI: small intestine.

7.4- and 11.0-fold in fundic and pyloric abomasa of resistant animals compared with susceptible ones, respectively (Fig. 3). However, this gene was unchanged in the small intestine. WSX-1 had the same expression profile as PIGR: over-expressed 3.6- and 19.9-fold in fundic and pyloric abomasa of resistant heifers, respectively, whereas no changes were observed in small intestine (Fig. 4).

4. Discussion

In this study, we compared cytokine mRNA expression profiles of resistant and susceptible heifers in response to an experimental challenge. With no changes in gene expression between resistant and susceptible animals at 3 DPI as expected, the profound increases in expression of pro-inflammatory cytokines/chemokines such as IL-1β, TNFα, and MIP-1α were observed at the sites of infection in resistant animals at 7 DPI. Even though pro-inflammatory cytokines/chemokines alone may not be sufficient to kill parasites, the change in micro-environment caused by release of nitric oxide, lytic enzymes, and reactive oxygen intermediates from activated macrophages could affect worm fecundity. The reduced fecundity, as indicated by the decreased number of eggs per female *Ostertagia* after previous exposures, seems to be a suitable parameter of acquired immunity (Claerebout and Vercruysse, 2000). Indeed, inflammatory cells and parasite-specific IgA in abomasum were inversely associated with *Haemonchus* worm burdens and EPG, indicating the cells and IgA may impair parasite development or fecundity in sheep (Amarante et al., 2004). Also, increase in these cytokines/chemokines leads to increased numbers of eosinophils being recruited into the abomasum and small intestines. The eosinophil-mediated killing as a possible effector mechanism for the elimination of L3 *Haemonchus contortus* larvae in immune sheep was observed (Rainbird et al., 1998). Indeed, the patterns of eosinophil recruitment that coincide with *Cooperia* adult worm expulsion has been documented (Kanobana et al., 2003).

Expression of IL-10 was also higher in resistant animals. IL-10 is an important immunomodulatory cytokine with pleiotropic functions. IL-10 has been considered a potent anti-inflammtory cytokine with the ability to inhibit the synthesis of pro-inflammatory cytokines such as TNFα, to suppress effect on Th1 lymphocytes/T cell activation, and to reduce antigen presentation. However, its immunostimulatory properties have been reported in human cancer (Mocellin et al., 2005). The observation that IL-10 production usually occurs concurrently with production of pro-inflammtory cytokines such as TNFα (Balloy et al., 2005; Mencacci et al., 1998) suggests that IL-10 may play a dampening role in keeping pro-inflammatory responses under control at the sites of infection. However, in small intestine where pro-inflammtory cytokines such as TNFα and IL-1β were not over-expressed in resistant heifers, IL-10 was also up-regulated, suggesting it may play a role in immunostimulation.

WSX-1, together with gp130, constitutes a functional receptor for IL-27, a heterodimeric cytokine related to IL-12 (Pflanz et al., 2002). Although its downstream signaling pathway has not been elucidated, WSX-1 is known to be required for IFN-γ production by naive $CD4^+$ T cells and essential for resistance to *Leishmania major* infection (Yoshida et al., 2001). WSX-1 plays a critical role in promoting Th1 development. WSX-1 knockout mice show higher levels of protective immunity against *Mycobacteria tuberculosis* infection than wild type mice (Holscher et al., 2005) with a concomitant increase of chronic inflammatory response. WSX-1 has been suggested as an inhibitory regulator of pro-inflammatory cytokine production (Yamanaka et al., 2004). The expression levels of WSX-1 and two components of its ligand IL-27, EBI3 and IL-27p28 were examined in draining mesenteric lymph node following *Trichuris muris* infection (Artis et al., 2004). WSX-1 remains unchanged 7 days post infection, which is consistent with our results in the draining lymph nodes. However, as controversial as it may seem, WSX knockout mice exhibit accelerated parasite expulsion mediated by enhanced production of Th2 cytokines (Artis et al., 2004). WSX-1 expression is markedly up-regulated in inflammatory $CD4^+$ T cells in an experimental disease model and regulation of its expression is cell-type dependent (Li et al., 2005). In the abomasum, WSX-1 was up-regulated up to ∼20-folds in resistant heifers, suggesting a potential role in rendering host resistance to nematodes. However, whether or not WSX-1 up-regulation is a cause or a consequence of over-expression of pro-inflammatory cytokines remains unclear.

Secretory IgA represents the first line of defense against pathogens at mucosal surfaces. A significant and negative correlation between the mucosal IgA levels and fecal egg counts and the number of eggs per female parasite has been reported, suggesting mucosal IgA may be associated with reduced *Ostertagia* fecundity (Claerebout and Vercruysse, 2000). An association between increased local IgA levels and decreased adult

Ostertagia circumcincta female worm length in sheep was observed (Smith et al., 1985; Strain and Stear, 1999). Although reduced worm length can result from inhibited growth or selective expulsion of large worms, worm length is an indicator of fecundity. No significant correlation was established between serum IgA and parasitological parameters expressing worm expulsion. However, the levels of *Cooperia*-specific IgA were significantly higher in intermediate responders than in low responders in cattle (Kanobana et al., 2001) and expulsion of the adult *Cooperia* worm appeared to be mast-cell independent and was associated with significant increase in mucosal IgA and influx of eosinophils (Kanobana et al., 2002). PIGR is the gene responsible for trans-epithelial transport of polymeric immunoglobulins such as IgA dimers and IgM pentamers into mucosal and glandular secretions. Its expression is essential for achieving mucosal immunity (Verbeet et al., 1995). Indeed, evidence demonstrated that PIGR knockout mice became more susceptible to *Mycobacteria bovis* bacillus Calmette-Guerin (BCG) infection (Tjarnlund et al., 2006). The reduced natural resistance to BCG was accompanied with reduced expression of protective mediators such as TNFα in this model. In mice absent of both passive and active immunity, expression of PIGR was significantly attenuated (Jenkins et al., 2003). Up-regulation of PIGR at both mRNA and protein levels following virus infection in human intestinal epithelial cells appeared to be an innate immune response against invading pathogen (Pal et al., 2005). Our results indicated PIGR was over-expressed in the abomasa of resistant heifers but remained unchanged in the small intestine. Although no parasite-specific IgA was monitored in our experiment, we do expect PIGR expression levels will be better correlated with mucosal IgA levels and subsequently with parasitological parameters. Together, these data suggest the local IgA response may be an important effector mechanism in the cattle–*Ostertagia* interaction.

In conclusion, we present evidence suggesting that resistant heifers can better maintain inflammatory responses at the sites of infection. The resistant heifers exhibited increased expression of PIGR in the abomasum, which could in turn increase trans-epithelial transport of IgA into secretory pathways, that allow mucus IgA to be one of the effector mechanisms against parasite infection. The difference in the cytokine profiles in the abomasum and the small intestine suggest that different parasites can induce distinct host immune responses. Our future work will include comparing the global gene expression profiles of eosinophils and macrophages as well as mast cells isolated by laser capture microdissection from the tissues of the resistant and susceptible heifers.

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